Tanshinone IIA may inhibit the growth of small cell lung cancer H146 cells by up-regulating the Bax/Bcl-2 ratio and decreasing mitochondrial membrane potential

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Abstract. Tanshinone IIA (Tan-IIA) may inhibit the growth of human non-small cell lung cancer A549 cells. However, the molecular mechanisms behind this malignancy have yet to be established. In the present study, we examined the effects of Tan-IIA on human small cell lung cancer H146 cells *in vitro*. The cytotoxicity of Tan-IIA in H146 cells was measured using the MTT assay. Mitochondrial membrane potential (MMP), reactive oxygen species (ROS) and Ca^{2+} in H146 cells were detected by flow cytometry, and the protein expression of Bax, Bcl-2, Caspase-3, NF-κBp65, GADD153 and β-actin in H146 cells was measured by Western blotting. H146 cells were inhibited in a dose-dependent manner. The protein expression of GADD153 and Caspase-3 was increased, but the proto-oncogene bcl-2 was notably decreased in H146 cells treated with Tan-IIA (5 µg/ml) for 24 h. FACS showed that Tan-IIA may increase the production of ROS and Ca^{2+}, but decreases MMP. The results indicate that Tan-IIA is capable of inhibiting the proliferation of H146 cells. One of the molecular mechanisms behind this effect may be the induction of ROS release and the decrease in MMP caused by an increase in the Bax/Bcl-2 ratio. Another may involve endoplasmic reticulum stress caused by the release of Ca^{2+} and an increase in GADD153 expression followed by a decrease in Bcl-2 expression, which induces a higher ratio of Bax/Bcl-2, in turn causing a decrease in MMP and leading to an increase in Caspase-3 expression and the inhibition of H146 cells. Thus, Tan-IIA may be a promising novel chemotherapeutic agent for the treatment of human small cell lung cancer H146 cells.

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Introduction

Danshen (*Salviae miltiorrhizae Radix*) was first recorded in the classical treatise Shen Nong Pen Tsao Ching (circa A.D. 100) (1), and is clinically administered to many cancer patients following chemotherapy or radiotherapy in Taiwan. However, its functions are controversial and have not been adequately explored. Lung cancer is the leading cause of cancer related death in Taiwan and in Western countries (2). However, the efficacy of chemotherapy for lung cancer remains unsatisfactory. Tanshinone IIA (Tan-IIA) (C19H18O3) is extracted from Danshen, *Salviae miltiorrhizae Radix* (3,4). There have been extensive investigations into the potential mechanisms of Tan-IIA as an anti-tumor agent in various tumor cells, including leukemia (5,6), breast cancer (7,8), colon cancer (9) and hepatocellular carcinoma cells (10,11). Tan-IIA may inhibit the proliferation of lung cancer NCI-H460 cells by decreasing Bcl-2 expression and inducing apoptosis (12). Our previous study also revealed that Tan-IIA inhibits the growth of non small cell lung cancer A549 cells by decreasing mitochondrial membrane potential (MMP) and increasing the ratio of Bax/Bcl-2 (13). However, the effects of Tan-IIA in human small cell lung cancer have yet to be established. In the present study, we investigated the molecular mechanisms of Tan-IIA in human small cell lung cancer H146 cells.

Materials and methods

Chemicals and reagents. Tan-IIA (Molecular Formula C19H18O3, >96% HPLC) was purchased from Herbasin Co. (Shenyang, China). Aprotinin, antipain, sodium deoxycholate, leupeptin, propidium iodide, sodium orthovanadate, Triton X-100, Tris-HCl, ribonuclease-A and MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO), potassium phosphates and TE buffer were purchased from Merck Co. (Darmstadt, Germany). RPMI 1640 medium, fetal bovine serum (FBS), penicillin-streptomycin, trypsin-EDTA, and glutamine were obtained from Gibco BRL (Grand Island, NY, USA). 10X SDS-PAGE running buffer, Tris, Tween 20, SDS, 5X TBE buffer were obtained from Amresco. (St. Louis, MO, USA);

BioMax Film was obtained from Kodak. DioC6 (84715) and DCFH-DA (C6827) were obtained from Biocompare (CA, USA). Anti-β-actin (MAB1501), anti-GADD153 and goat anti-mouse IgG horseradish peroxidase conjugated antibody (AP124P) were obtained from Chemicon (MA, USA). Anti-Bcl-2 (N-19-sc-492) and anti-Bax (N-19-sc-492) were from Santa Cruz Biotechnology, Inc.

Cell line and culture. Human lung cancer H146 cells were obtained from the Food Industry Research and Development Institute (Hsinchu, Taiwan). The H146 cells were placed in 25-cm^2 tissue culture flasks and maintained in RPMI 1640 containing 10% heat-inactivated FBS (Gibco BRL, Grand Island, NY, USA), 100 U/ml penicillin and 100 μg/ml strepto-mycin. Cells were grown at 37˚C in a humidified atmosphere of 95% air and 5% CO_2. All data presented are from at least three independent experiments.

Determination of cell viability. The H146 cells were plated at a density of 1x10^5 cells/well and grown for 24 h. Various concentrations of Tan-IIA were added and the cells were grown for 24, 48 and 72 h. For the control regimen, DMSO 0.2% (solvent) was used. After 24, 48 and 72 h of culture, cell viability was evaluated by the MTT assay (14), conducted in triplicate. Briefly, H146 cells were seeded in a 6-well plate at a density of 1x10^5 cells/well and allowed to adhere overnight. After removing the medium, 2,000 μl of fresh medium per well, containing 10 mM HEPES and 200 μl of MTT, was added to the wells. The plate was incubated for 30 min at 37˚C in the dark, then the medium was removed and 1,000 μl of DMSO was added to the wells. Absorbance was measured using an ELISA plate reader at 590 nm.

Detection of reactive oxygen species. The levels of reactive oxygen species (ROS) in the H146 cells were examined and determined by flow cytometry (Becton Dickinson FACSCalibur) as previously described (13). Briefly, ~2x10^5 cells/well of H146 cells in 12-well plates were incubated with or without Tan-IIA for different time periods (0, 6, 12 and 24 h) to detect changes in ROS. The cells were harvested and washed twice, re-suspended in 500 μl of 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA) (10 μM) and incubated at 37˚C for 30 min, then analyzed by flow cytometry.

Detection of mitochondrial membrane potential. The mito-chondrial membrane potential (MMP) levels in H146 cells were determined by flow cytometry (Becton Dickinson FACSCalibur) as previously described (13). Briefly, ~2x10^5 cells/well of H146 cells in 12-well plates were incu-bated with or without Tan-IIA for different time periods (0, 6, 12 and 24 h) to determine changes in MMP. Cells were harvested and washed twice, re-suspended in 500 μl of DiOC6 (4 μmol/l) and incubated at 37˚C for 30 min, then analyzed by flow cytometry.

Determination of Ca^{2+} concentrations. Ca^{2+} levels in the H146 cells were determined by flow cytometry (Becton Dickinson FACSCalibur) as previously described (13). Briefly, ~2x10^5 cells/well of H146 cells in 12-well plates were incubated
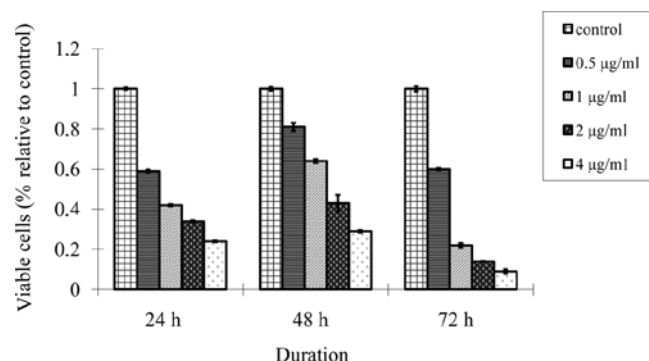

Figure 1. H146 cells were treated with increasing Tan-IIA concentrations of 0, 0.5, 1, 2 and 4 μg/ml for different time periods (24, 48 and 72 h). The cytotoxicity of Tan-IIA in H146 cells was determined using the MTT assay. Tan-IIA significantly inhibited H146 cell growth. Each point is the mean ± SD of three experiments. *P<0.05.

with or without Tan-IIA for different time periods (0, 6, 12 and 24 h) to detect changes in Ca^{2+} levels. Cells were harvested and washed twice, re-suspended in Indo 1/AM (3 μg/ml), incubated at 37˚C for 30 min, then analyzed by flow cytometry.

Western blotting. To determine the effect of Tanshinone IIA on P53, Bcl-2, Bax, Caspase-3, GADD153, NF-κBp65 and β-actin levels in H146 cells, Western blotting was used. After treatment with or without various concentrations of Tan-IIA (0, 1, 2 and 5 μg/ml) for 24 h, total proteins were collected from H146 cells and GADD153 and β-actin levels were examined by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting, as described previously (15,16). After treatment with or without various concentrations of Tan-IIA (0, 1, 2.5 and 5 μg/ml) for 24 h, total proteins were collected from H146 cells and P53, Bcl-2, Bax, Caspase-3, NF-κBp65 and β-actin levels were examined by SDS-PAGE and Western blotting, as described previously (15,16).

Statistical analysis. Values are presented as the percentage (mean ± SD) of the control. The Student's t-test was used to analyze the statistical significance between the Tan-IIA-treated and control groups. A P-value of <0.05 was considered significant for all tests.

Results

Cytotoxic effects of Tanshinone IIA in H146 cells. H146 cells were cultured with various concentrations of Tan-IIA (control, 0.5, 1, 2 and 4 μg/ml) for different time periods (24, 48 and 72 h), then the cytotoxicity of Tan-IIA in H146 cells was determined using the MTT assay. The percentage of viable cells (relative to the control) was 58.97±0.53, 41.59±0.25, 33.57±0.2 and 23.64±0.1%, respectively, at 24 h, 81.22±1.05, 64.45±0.4, 42.56±1.12 and 29.11±0.1%, respectively, at 48 h and 60.27±0.61, 22.32±0.46, 14.39±0.06 and 8.67±0.17%, respectively, at 72 h. The IC$_{50}$ was 0.82, 0.96 and 0.69 μg/ml, respectively, for Tan-IIA to affect H146 cells at different time periods of treatment (24, 48 and 72 h). The results indicate that Tan-IIA inhibited the proliferation of H146 cells in a dose-dependent manner (Fig. 1).
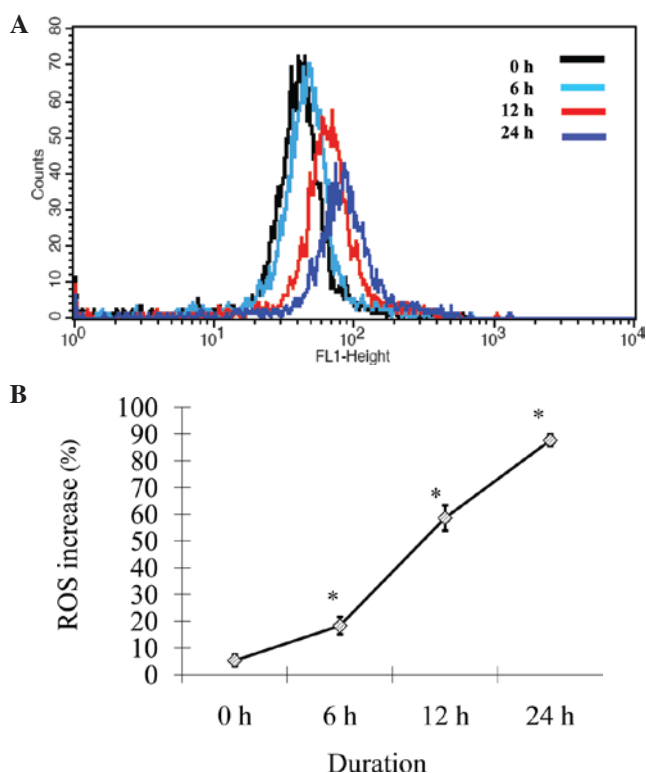
Figure 2. Flow cytometric analysis of reactive oxygen species (ROS) in H146 cells treated with Tan-IIA. Approximately 2×10^5 cells/well of H146 cells in 12-well plates were incubated with Tan-IIA for different time periods (0, 6, 12, and 24 h) to detect changes in ROS. The percentage of cells stained by DCFH-DA dye was determined by flow cytometry. Each point is the mean ± SD of three experiments. $^*P<0.05$.
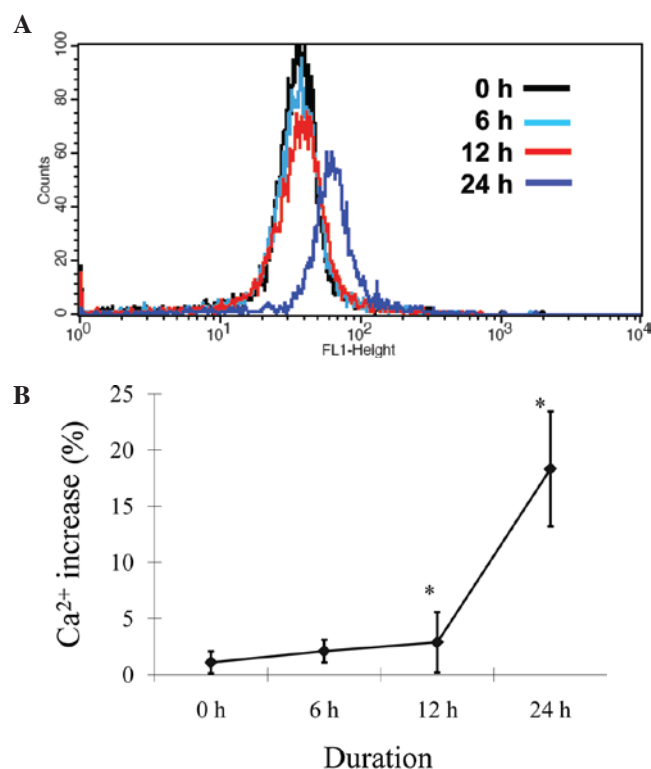

Figure 4. Flow cytometric analysis of Ca^{2+} concentration in H146 cells treated with Tan-IIA. Approximately 2×10^5 cells/well of H146 cells in 12-well plates were incubated with Tan-IIA for different durations (0, 6, 12, and 24 h) to detect changes in Ca^{2+} levels. The percentage of cells that were stained by Indo-1/AM dye was determined by flow cytometry. Each point is the mean ± SD of three experiments. $^*P<0.05$.
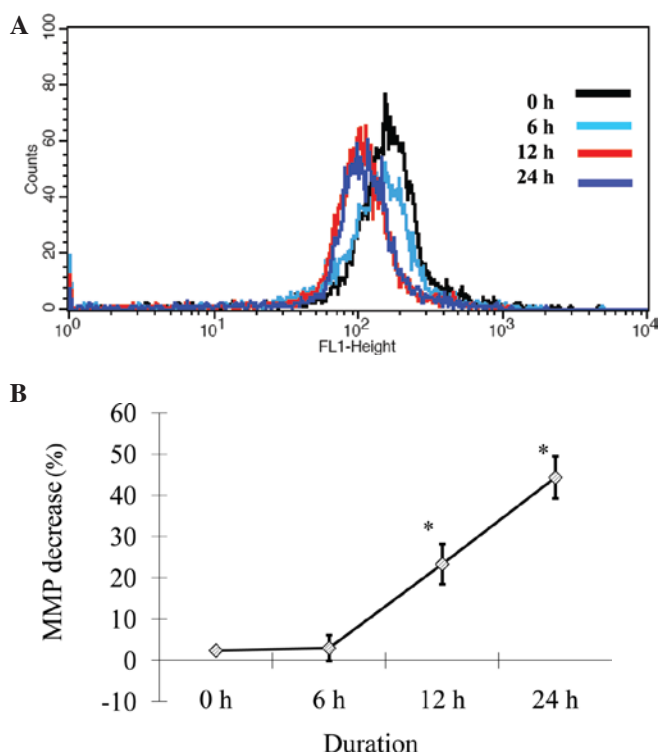

Figure 3. Flow cytometric analysis of mitochondrial membrane potential (MMP) in H146 cells treated with Tan-IIA. Approximately 2×10^5 cells/well of H146 cells in 12-well plates were incubated with Tan-IIA for different time periods (0, 6, 12, and 24 h) to determine changes in MMP. The percentage of cells stained by DiOC6 dye was determined by flow cytometry. Each point is the mean ± SD of three experiments. $^*P<0.05$.

Tanshinone IIA induced the production of reactive oxygen species in H146 cells. Following treatment of the H146 cells with or without 1 μg/ml of Tan-IIA for various time periods (0, 6, 12 and 24 h), the increasing percentages of ROS were 5.32±2.11, 18.32±3.25, 58.62±4.78 and 87.66±2.14%, respectively. Increasing the co-treated time periods resulted in an increase in ROS release (Fig. 2A and B). These results showed that Tan-IIA promoted ROS production in a time-dependent manner.

Tanshinone-IIA suppressed mitochondrial membrane potential in H146 cells. H146 cells were treated with or without Tan-IIA (1 μg/ml) for various time periods (0, 6, 12 and 24 h), then the MMP was analyzed by flow cytometry. The decreasing percentages of the MMP were 2.38±0.19, 2.98±3.14, 23.32±4.87 and 44.33±5.10%, respectively. Increasing the duration of incubation led to the decrease of MMP in the H146 cells (Fig. 3A and B).

Tanshinone IIA induced Ca^{2+} production in H146 cells. H146 cells were treated with or without 1 μg/ml Tan-IIA for various time periods (0, 6, 12 and 24 h). The increasing percentages of Ca^{2+} concentrations were 1.09±0.98, 2.09±1.00, 2.87±2.69 and 18.32±5.12%, respectively. Increasing the duration of incubation led to an increase in the concentration of Ca^{2+} in H146 cells (Fig. 4A and B).

Effect of Tanshinone IIA on NF-κBp65, Bcl-2, Bax, Caspase-3, GADD153 and β-actin levels by Western blotting. H146 cells (5×10^6/ml) were treated with various concentrations of Tan-IIA (1, 2 and 5 μg/ml) for 24 h. Once the total proteins

A

B

A

B

C

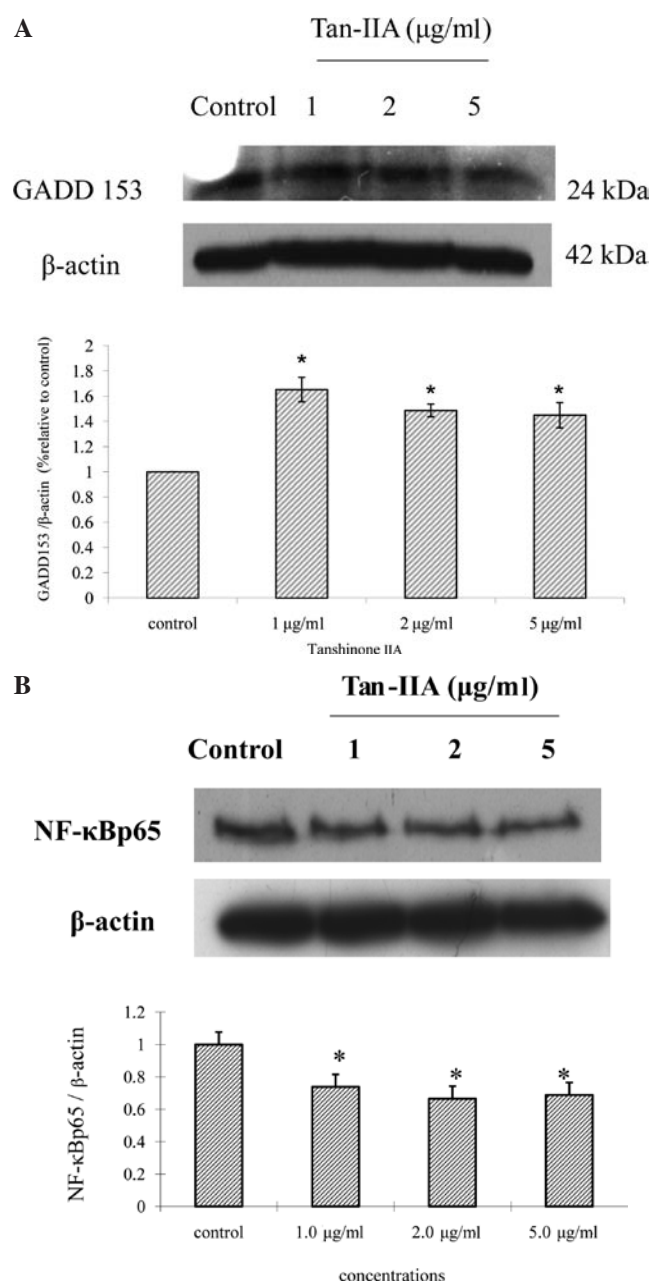Figure 5. Protein expression of GADD153, NF-κBp65 and β-actin in H146 cells. H146 cells (5×10^6/ml) were treated with various concentrations of Tan-IIA (0, 1, 2 and 5 μg/ml) for 24 h before being harvested by centrifugation. Total protein was prepared and determined, then levels of GADD153 and β-actin expression were estimated by Western blotting. Protein expression of GADD153 was significantly increased after treatment with Tan-IIA (Fig. 5A). Tan-IIA decreased the protein expression of NF-κBp65 in H146 cells at the concentrations of 1, 2 and 5 μg/ml 24 h (Fig. 5B). Each point is the mean ± SD of three experiments. $^*P<0.05$.

were prepared and determined, the levels of GADD153 and β-actin expression were estimated by Western blotting. Tan-IIA increased the protein expression of GADD153 in the H146 cells at concentrations of 1, 2 and 5 μg/ml at 24 h (Fig. 5A). Tan-IIA decreased the protein expression of NF-κBp65 in the H146 cells at concentrations of 1, 2 and 5 μg/ml at 24 h (Fig. 5B). The H146 cells (5×10^6/ml) were treated with various concentrations of Tan-IIA (1, 2.5 and 5 μg/ml) for 24 h. After the total proteins were prepared and determined, the levels of Bax and Bcl-2 expression were estimated by Western blotting. The protein expression of Bax (Fig. 6A) was mildly decreased
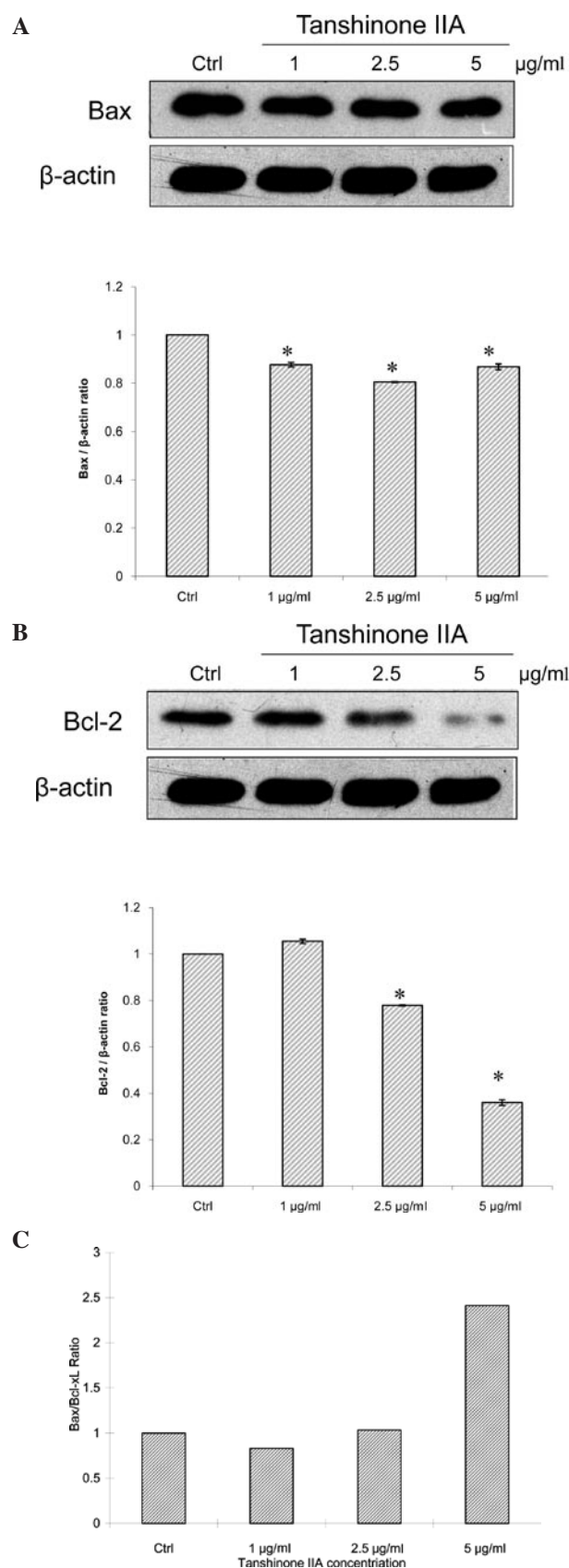
Figure 6. Protein expression of Bax, Bcl-2 and β-actin in H146 cells. H146 cells (5×10^6/ml) were treated with various concentrations of Tan-IIA (1, 2.5 and 5 μg/ml) for 24 h. Total protein was prepared and determined, then levels of Bax and Bcl-2 expression were estimated. The protein expression of Bax was mildly decreased after treatment with Tan-IIA for 24 h (Fig. 6A). The protein expression of Bcl-2 was significantly decreased after treatment with Tan-IIA for 24 h (Fig. 6B), while the ratio of Bax/Bcl-2 was significantly increased (Fig. 6C). Each point is the mean ± SD of three experiments. $^*P<0.05$.
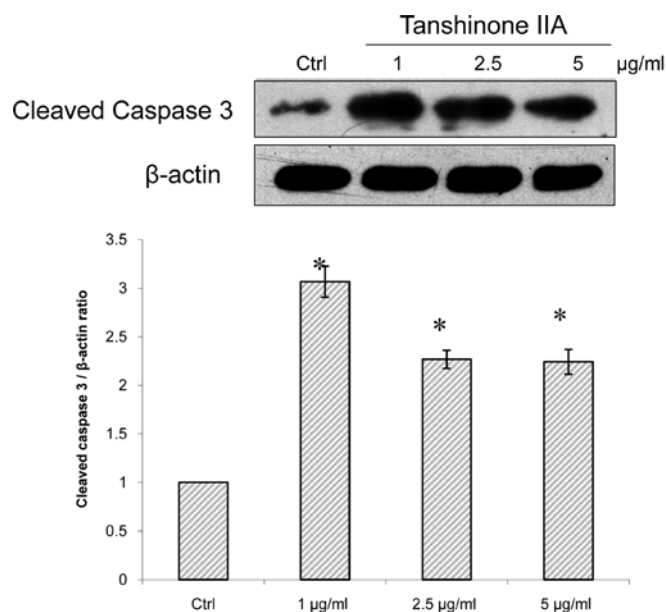
Figure 7. Protein expression of Caspase-3 and β-actin in H146 cells. The H146 cells (5x10⁶/ml) were treated with various concentrations of Tan-IIA (1, 2.5 and 5 μg/ml) for 24 h. Total protein was prepared and determined, then levels of Caspase-3 expression were estimated by Western blotting. The protein expression of Caspase-3 was `significantly increased after treatment with Tan-IIA for 24 h. Each point is the mean ± SD of three experiments. *P<0.05.
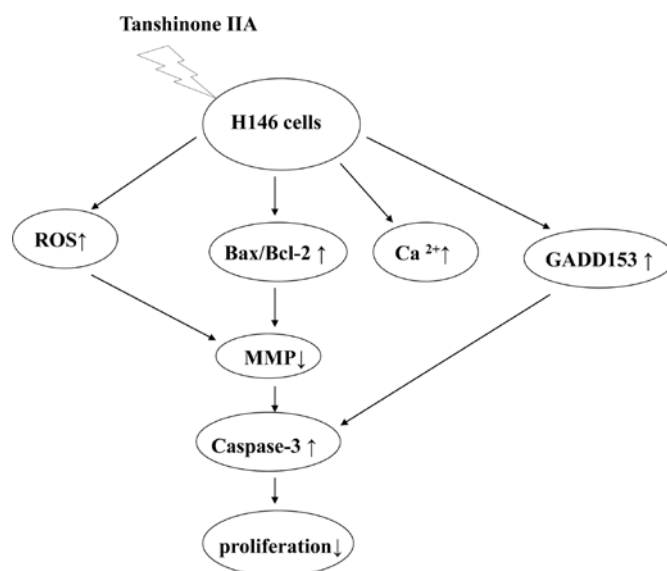

Figure 8. Proposed model for the molecular mechanisms of Tan-IIA inhibition of the proliferation of H146 cells. One of the molecular mechanisms may be through the induction of ROS release and the decrease in MMP due to an increase in the Bax/Bcl-2 ratio. Another may be through ER stress caused by the release of Ca²⁺ and an increase in GADD153 expression followed by a decrease in Bcl-2 expression, leading to the induction of a higher ratio of Bax/Bcl-2 and then a decrease in MMP, resulting in increased Caspase-3 expression and the inhibition of H146 cells.

after treatment with Tan-IIA for 24 h. The protein expression of Bcl-2 (Fig. 6B) was significantly decreased after treatment with Tan-IIA for 24 h, but the ratio of Bax/Bcl-2 was significantly increased (Fig. 6C). The protein expression of Caspase-3 was significantly increased after treatment with Tan-IIA for 24 h (Fig. 7).

Discussion

The results of the present study indicate that Tan-IIA may inhibit the proliferation of small cell lung cancer H146 cells in a dose-dependent manner. The mitochondria have been shown to be the central control point of apoptosis (17). It is well documented that decreases in MMP induce apoptosis (18). The overexpression of Bax may decrease MMP; however, it is well known that the overexpression of Bcl-2 prevents the decrease of MMP (19). Tan-IIA may increase Caspase-3 protein expression in H146 cells. This is in agreement with a previous study (12). Our results show that Tan-IIA decreased the protein expression of Bax and Bcl-2. This is not in agreement with a previous study that showed that Tan-IIA up-regulated Bax but down-regulated Bcl-2 protein expression in SPC-A-1 cells (20). In the present study, the results revealed that Tan-IIA increased the ratio of Bax/Bcl-2 and decreased MMP significantly. Tan-IIA increased ROS generation significantly after 6 h, but decreased MMP significantly after 12 h. On the basis of these results, we suggest that Tan-IIA may act through the induction of ROS and by increasing the ratio of Bax/Bcl-2, leading to a decrease in MMP, which in turn induces apoptosis in H146 cells. Endoplasmic reticulum (ER) stress is one apoptotic cell death pathway. It is well documented that the overexpression of GADD153 represents a new and important strategy in anticancer therapy (21). GADD153 is indicative of DNA damage and ER stress, and

was promoted in previous studies, where it induced apoptosis (22). Several studies have shown that GADD153 suppresses the activation of Bcl-2 (23,24). Our results also reveal that Tan-IIA is capable of increasing the protein expression of GADD153 in H146 cells. On the other hand, the marked release of Ca²⁺ was observed in the H146 cells at 24 h. One of the molecular mechanisms for the Tan-IIA inhibition of H146 cells may be through ER stress caused by the release of Ca²⁺ and an increase in the protein expression of GADD153 followed by a decrease in Bcl-2 protein expression, which induces a higher ratio of Bax/Bcl-2 then a decrease in MMP, leading to an increase in the protein expression of Caspase-3 and the inhibition of the proliferation of H146 cells. This proposed model is the first report regarding the action of Tan-IIA in small cell lung cancer H146 cells (Fig. 8). Tan-IIA appears to be a promising new chemotherapeutic agent for small cell lung cancer.

Acknowledgements

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